

## Effects of divalent cations on exocytosis and endocytosis from single mouse pancreatic $\beta$ -cells

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1. The effects of the divalent cations  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  on exocytosis and endocytosis from single isolated mouse pancreatic  $\beta$ -cells were investigated by monitoring changes in cell capacitance.
2. The immediate increase in capacitance elicited by a single depolarization from  $-70$  to  $+20$  mV was dependent on the divalent cation species, with  $\text{Ca}^{2+}$  ( $8.2 \pm 1.1$  fF  $\text{pC}^{-1}$ )  $>$   $\text{Ba}^{2+}$  ( $1.0 \pm 0.2$  fF  $\text{pC}^{-1}$ )  $>$   $\text{Sr}^{2+}$  ( $0.7 \pm 0.2$  fF  $\text{pC}^{-1}$ ) in perforated-patch recordings.
3. In  $\text{Ba}^{2+}$  solutions alone there was subsequently an additional slow increase in capacitance (to  $4.3 \pm 1.1$  fF  $\text{pC}^{-1}$ ). This second phase of exocytosis was unaffected by preincubation with colcemid ( $20 \mu\text{M}$ , 45 min) or cytochalasin D ( $10 \mu\text{M}$ , 15 min), suggesting that interaction of secretory granules with microtubules or microfilaments is not involved.
4. An increase in cell capacitance was elicited by depolarization in  $\text{Ba}^{2+}$  solutions when intracellular  $\text{Ca}^{2+}$  was buffered with  $10$  mM EGTA. Infusion of the  $\beta$ -cell with  $\text{Ba}^{2+}$  also stimulated exocytosis although the rate was much slower ( $1.1 \pm 0.2$  fF  $\text{s}^{-1}$ ;  $8 \mu\text{M}$  free  $\text{Ba}^{2+}$ ) than for  $\text{Ca}^{2+}$  ( $39 \pm 5$  fF  $\text{s}^{-1}$ ;  $2 \mu\text{M}$  free  $\text{Ca}^{2+}$ ). These data indicate that  $\text{Ba}^{2+}$  does not evoke secretion by promoting  $\text{Ca}^{2+}$  release from internal stores.
5. The lower efficacy of  $\text{Ba}^{2+}$  in supporting exocytosis may be related to the fact that this cation does not activate calmodulin-dependent processes and the slow second phase of secretion may result from this ion being removed only slowly from the cytoplasm.
6. Endocytosis was faster in  $\text{Sr}^{2+}$  than in  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$  solution, and the speed increased when the external concentration of all three divalent cation species was raised. The ability of  $\text{Ba}^{2+}$  to support endocytosis suggests calmodulin-dependent processes are not involved. These data suggest membrane retrieval is regulated differently from exocytosis in  $\beta$ -cells.

The divalent cations  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  can replace  $\text{Ca}^{2+}$  in a variety of biological processes. In pancreatic  $\beta$ -cells,  $\text{Ba}^{2+}$  acts as a potent primary secretagogue and is able to release insulin in the absence of glucose (Hales & Milner 1968; Wollheim, Blondel, Trueheart, Renold & Sharp, 1975; Somers, Devis, Obberghen & Malaisse, 1976). This effect of  $\text{Ba}^{2+}$  is suppressed by agents which block voltage-gated  $\text{Ca}^{2+}$  channels (Somers *et al.* 1976), suggesting it results, at least in part, from the ability of  $\text{Ba}^{2+}$  to block the ATP-sensitive  $\text{K}^{+}$  channel (Quayle, Standen & Stanfield, 1988; M. Takano & F. M. Ashcroft, unpublished observations) and thereby produce depolarization, activation of voltage-gated  $\text{Ca}^{2+}$  influx and insulin secretion (Ashcroft & Rorsman, 1989). Since  $\text{Ba}^{2+}$  is able to elicit insulin release in  $\text{Ca}^{2+}$ -free solutions (Hales & Milner, 1968), the cation must also be able to substitute for  $\text{Ca}^{2+}$  in the exocytotic process.  $\text{Sr}^{2+}$  does not support insulin secretion in the absence of glucose but is able to replace  $\text{Ca}^{2+}$  in sustaining glucose-stimulated

insulin release, although it is not as effective (Henquin, 1980). In this paper we have investigated the effects of  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  as charge carriers and as stimulators of exocytosis, in single voltage-clamped  $\beta$ -cells to determine whether differences in the amount of divalent cation entry, or in the efficacy of the divalent cation to support exocytosis, account for the differential ability of  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$  to sustain insulin secretion. We have used the capacitance technique (Neher & Marty, 1982; Joshi & Fernandez, 1988; Ämmälä, Eliasson, Bokvist, Larsson, Ashcroft & Rorsman, 1993*b*) to monitor exocytosis and endocytosis from single  $\beta$ -cells with high temporal resolution. We have shown elsewhere that the increase in  $\beta$ -cell capacitance elicited by depolarization is associated with the release of secretory granule contents, indicating that the capacitance increase represents exocytosis (Smith, Proks & Ashcroft, 1994).

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## METHODS

### Cell preparation

NMRI mice were killed by cervical dislocation, and pancreatic islets isolated by collagenase digestion and dispersed into single  $\beta$ -cells by low- $\text{Ca}^{2+}$  treatment, as previously described (Rorsman & Trube 1986; Åmmälä *et al.* 1993b). Cells were plated onto Petri dishes (Falcon) and maintained for 1–5 days in RPMI tissue culture medium (Life Technologies Inc., Paisley, UK) supplemented with 10% fetal calf serum, 10 units  $\text{ml}^{-1}$  penicillin and 10  $\mu\text{g ml}^{-1}$  streptomycin and gassed with 95% air–5%  $\text{CO}_2$ . Cells were used within 3 days of isolation.

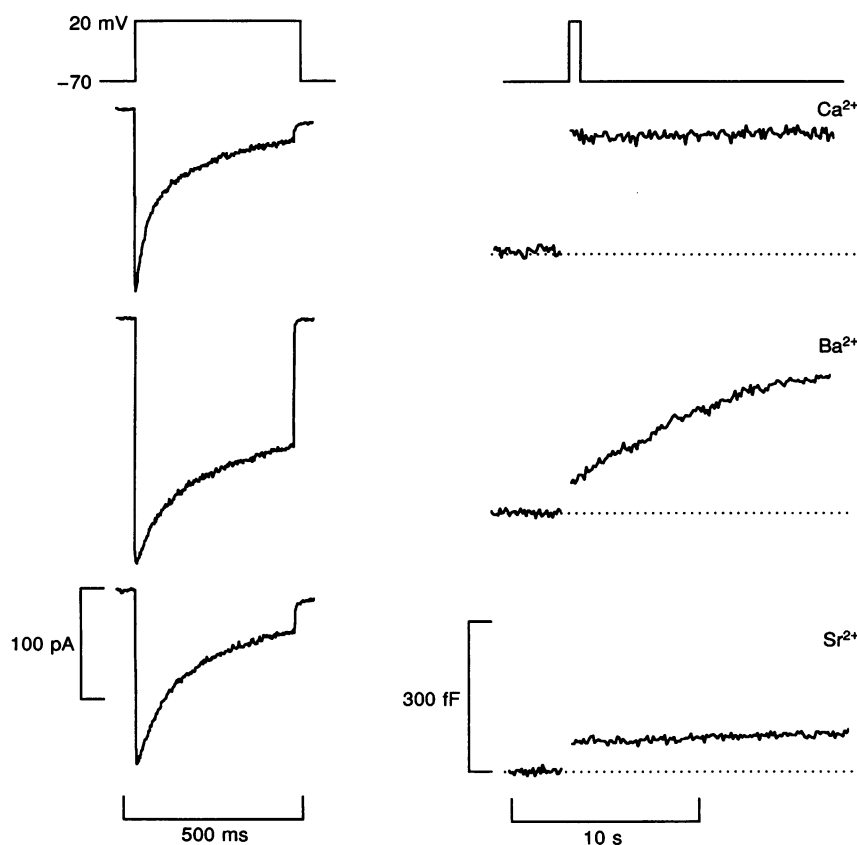
### Recording methods

Patch pipettes were pulled from 1.5 mm borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany), coated with Sylgard to reduce their electrical capacitance and fire polished immediately before use. They had resistances of between 3 and 6  $\text{M}\Omega$  when filled with pipette solution.

Whole-cell currents and changes in cell capacitance were recorded using an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). The zero current potential of the pipette was adjusted before establishment of the seal, with the pipette in the bath, and no corrections have been made for liquid junction potentials. The

mean cell capacitance at the start of the experiment averaged 4 pF and experiments were initiated once the series conductance exceeded 30 nS. Changes in cell capacitance were measured as described previously (Åmmälä *et al.* 1993b). Briefly, a 20 mV root mean square (r.m.s.) 800 Hz sine wave was added to the holding potential (Joshi & Fernandez, 1988; Fiddler Lim, Nowycky & Bookman, 1990) and ten cycles were averaged for each data point. The resulting current was analysed at two orthogonal phase angles with a resolution of 100 ms per point. The phase angle was determined empirically for each experiment by varying the  $C_{\text{slow}}$  and  $G_{\text{series}}$  knobs on the amplifier, and was periodically checked throughout the experiment. Capacitance and conductance changes were calibrated using a 300 fF capacitance step and a 10–30 nS conductance step. Capacitance changes were evoked by interrupting the sinusoidal wave and applying a depolarizing pulse from a holding potential of  $-70$  mV. Measurements were carried out using a Digidata A/D converter (Axon Instruments, Burlingame, CA, USA), a Viglen 486 computer and in-house software written in AxoBasic (Axon Instruments). Charge entry during a depolarizing voltage step was calculated by integrating the inward current assuming a baseline of 0 pA.

All data are presented as means  $\pm$  standard error of the mean ( $n$  = number of cells).



**Figure 1. Effects of divalent cations on the capacitance response to a single pulse**

Whole-cell currents (left) and changes in corresponding cell capacitance (right) evoked by a 500 ms depolarization to  $+20$  mV from a holding potential of  $-70$  mV in the presence of 5 mM each of extracellular  $\text{Ca}^{2+}$  (top traces),  $\text{Ba}^{2+}$  (middle traces) and  $\text{Sr}^{2+}$  (bottom traces). Here and in subsequent figures, the pulse protocol is shown above the current records, and the dotted line indicates the prestimulatory capacitance level. Note that the time scale is different for the current and capacitance traces.

## Solutions

The standard extracellular (bath) solution contained (mM): 140 NaCl, 5.6 KCl, 1.2 MgCl<sub>2</sub>, 10 Hepes (adjusted to pH 7.4 with NaOH), 5 glucose, 20 TEA (to block voltage-activated K<sup>+</sup> currents) and 5 CaCl<sub>2</sub>, BaCl<sub>2</sub>, or SrCl<sub>2</sub>. Higher divalent cation concentration solutions were obtained by addition of the chloride salt to this extracellular solution. Most experiments were carried out on intact cells using the perforated-patch recording configuration. For these experiments, 10  $\mu$ M forskolin was added to the bath solution (to increase the secretory response; Ämmälä, Ashcroft & Rorsman, 1993a) and the pipette solution contained (mM): 76 Cs<sub>2</sub>SO<sub>4</sub> (to reduce outward K<sup>+</sup> currents), 10 NaCl, 10 KCl, 1 MgCl<sub>2</sub> and 5 Hepes (pH 7.35 with CsOH). Electrical contact was established by the addition of amphotericin B to the pipette solution (final concentration, 240  $\mu$ g ml<sup>-1</sup> in 0.4% DMSO) as previously described (Ämmälä *et al.* 1993b; Smith, Ashcroft & Fewtrell, 1993). Voltage clamp was regarded as satisfactory when the series conductance was >30 nS. For standard whole-cell recordings the pipette was filled with (mM): 125 potassium glutamate, 1 MgCl<sub>2</sub>, 3 MgATP, 0.1 cyclic AMP, 5 Hepes (pH 7.15 with KOH) and either 10 EGTA + 1 BaCl<sub>2</sub> (for 8  $\mu$ M free Ba<sup>2+</sup>) or 10 EGTA + 9 CaCl<sub>2</sub> (for 2  $\mu$ M free Ca<sup>2+</sup>).

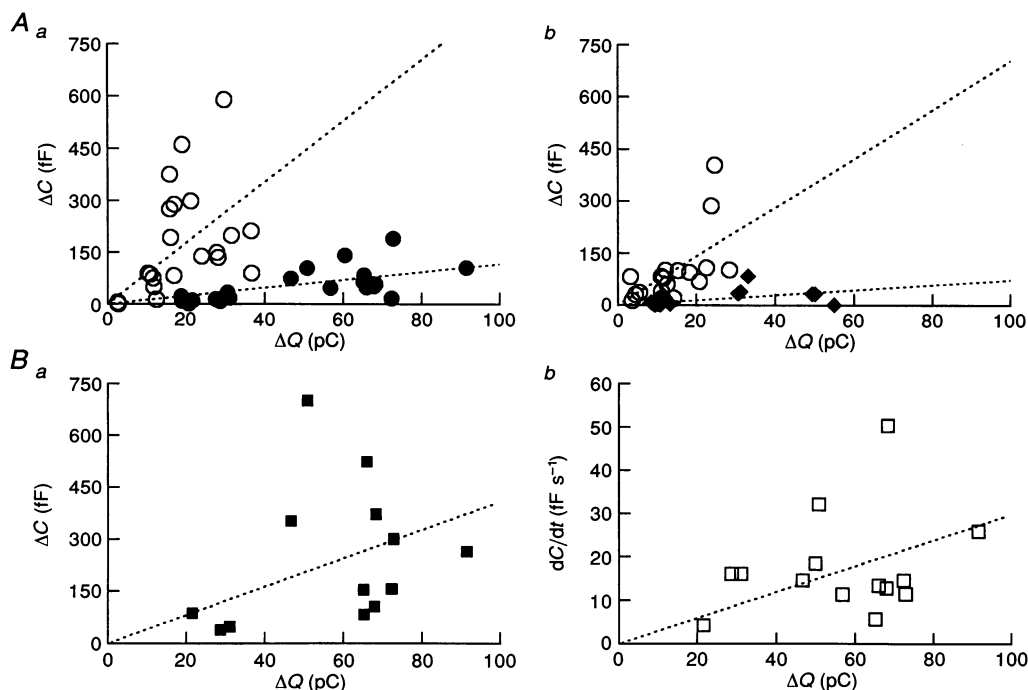
Cytochalasin D (Sigma) was prepared from a 2 mM stock solution in DMSO and was added to the bath solution (final DMSO concentration, 0.5%). Colcemid (Sigma) was prepared immediately before the experiment from a 27 mM stock solution in water (stored at -20 °C).

The bath was continuously perfused and complete exchange was achieved within 1–2 min. All experiments were carried out at 30–32 °C.

## RESULTS

### Capacitance changes elicited by a single depolarization

We first explored the effects of divalent cations on the exocytotic response to a single voltage pulse. In each case, cells were first exposed to Ca<sup>2+</sup>-containing external solution and then to one containing either Ba<sup>2+</sup> or Sr<sup>2+</sup>. Figure 1 shows an example of the effect of Ca<sup>2+</sup>, Ba<sup>2+</sup> and Sr<sup>2+</sup> on the whole-cell currents and associated changes in cell capacitance produced by a single depolarization to +20 mV.



**Figure 2.** Relationship between the amount of divalent cation entry and the capacitance response

A, relationship between the increase in cell capacitance ( $\Delta C$ ) and the amount of charge entry ( $\Delta Q$ ) during a single 500 ms pulse from -70 to +20 mV in cells exposed first to 5 mM Ca<sup>2+</sup> (○) and then to 5 mM Ba<sup>2+</sup> (●) (Aa), and cells first exposed to 5 mM Ca<sup>2+</sup> (○) and then to 5 mM Sr<sup>2+</sup> (◆) (Ab). The dashed lines represent the best fit of the data, using least-squares analysis, to the equation  $y = ax$ , where  $a$  is the slope of the relationship (stated in the text). Both those cells which did, and those which did not, exhibit an increase in capacitance after the depolarization in Ba<sup>2+</sup> solution were included in this analysis. B, analysis of the slow increase in capacitance following a single 500 ms pulse from -70 to +20 mV in 5 mM Ba<sup>2+</sup> solution. Ba, the relationship between the total cell capacitance change ( $\Delta C$ ) and the amount of charge entry ( $\Delta Q$ ). Bb, the relationship between the initial speed of the capacitance change ( $dC/dt$ ), measured during the first second after the pulse, and charge entry ( $\Delta Q$ ). The dashed lines represent the best fit to the data by least-squares analysis. Only cells which exhibited an increase in capacitance after the depolarization in Ba<sup>2+</sup> solution were included in this analysis.

It is clear that although the inward currents were larger in  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solutions, the capacitance increase observed immediately after cessation of the voltage pulse was much smaller than in  $\text{Ca}^{2+}$  solution. The mean integrated charge was  $19.0 \pm 2.2$  ( $n = 39$ ),  $49.2 \pm 5.0$  ( $n = 20$ ) and  $20.8 \pm 3.8$  pC ( $n = 18$ ), in  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solution, respectively, whereas the mean change in capacitance was  $155 \pm 20$ ,  $49 \pm 5$  and  $16 \pm 5$  fF, respectively. Even when expressed as a fraction of the integrated current, to correct for the observed difference in charge entry, exocytosis varied with the divalent cation, being  $8.2 \pm 1.1$ ,  $1.0 \pm 0.2$  and  $0.7 \pm 0.2$  fF pC $^{-1}$ , in  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solution, respectively. There was no further increase in exocytosis following the voltage step in  $\text{Ca}^{2+}$  or  $\text{Sr}^{2+}$  solutions and the capacitance trace remained flat. When  $\text{Ba}^{2+}$  carried the inward current, however, in about 60% of all cells (12 out of 20) the capacitance continued to increase slowly after the pulse (Fig. 1, middle panel). The total increase in capacitance elicited by a single depolarization in  $\text{Ba}^{2+}$  solution amounted to  $245 \pm 57$  fF or  $4.3 \pm 1.1$  fF pC $^{-1}$  ( $n = 12$ ), a value still less than that obtained in  $\text{Ca}^{2+}$  solution.

Figure 2A compares the relationship between the amount of charge entry and the immediate capacitance change evoked by a single depolarization in  $\text{Ba}^{2+}$  (Fig. 2A *a*) and  $\text{Sr}^{2+}$  (Fig. 2A *b*) solutions with that measured under control conditions in  $\text{Ca}^{2+}$  solution. Although there is considerable scatter in the data it is clear that for all three cations exocytosis increases with increasing charge entry. The slope of the relationship, however, is less in  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solutions than in  $\text{Ca}^{2+}$  solution, being  $1.2 \pm 0.15$  fF pC $^{-1}$  in

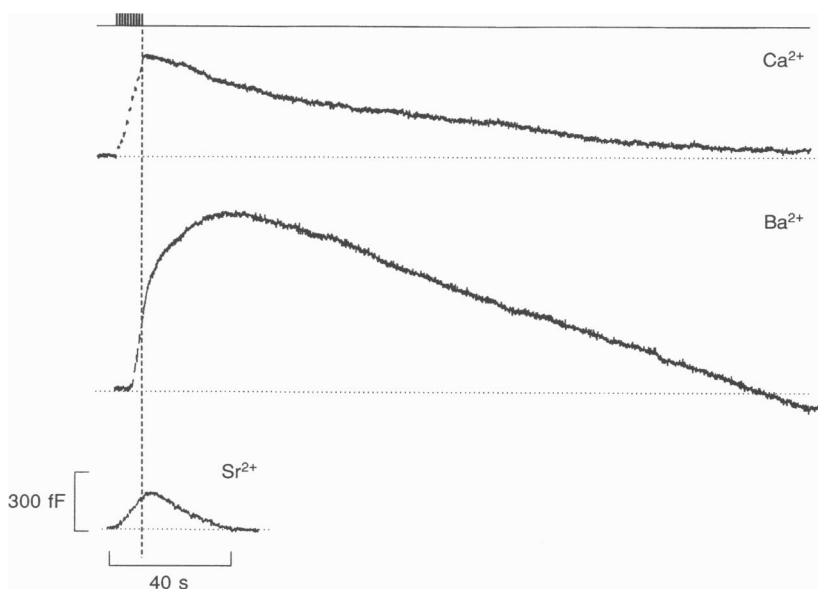
$\text{Ba}^{2+}$  compared with  $8.8 \pm 1.6$  fF pC $^{-1}$  in  $\text{Ca}^{2+}$  (Fig. 2A *a*,  $P < 0.25$ ), and  $0.7 \pm 0.18$  fF pC $^{-1}$  in  $\text{Sr}^{2+}$  compared with  $7.1 \pm 1.4$  fF pC $^{-1}$  in  $\text{Ca}^{2+}$  (Fig. 2A *b*,  $P < 0.3$ ), when measured in the same cells. These results indicate that  $\text{Ca}^{2+} > \text{Ba}^{2+} > \text{Sr}^{2+}$  in supporting the initial secretory response to a single depolarization.

The second phase of exocytosis observed in  $\text{Ba}^{2+}$  solutions is analysed more fully in Fig. 2B. The total increase in capacitance elicited by the pulse, i.e. the immediate increase plus the slow phase (Fig. 2B *a*), and the rate of the capacitance increase measured during the first second after the end of the pulse (Fig. 2B *b*) are plotted as a function of  $\text{Ba}^{2+}$  entry during the preceding pulse. Although there appears to be a general trend towards an increase in both the extent and rate of exocytosis with increasing  $\text{Ba}^{2+}$  entry, the data are scattered and there is no significant correlation.

Most of the secretory response to a single depolarization which occurs in  $\text{Ba}^{2+}$  solution results from the slow phase of exocytosis (in those cells where it is present). The mean initial increase in capacitance was  $1.0 \pm 0.2$  fF pC $^{-1}$  ( $n = 20$ ), whereas that during the slow phase was  $3.3 \pm 0.7$  fF pC $^{-1}$  ( $n = 12$ ). The mean rate of exocytosis during the first second after the end of the pulse was  $18 \pm 3$  fF s $^{-1}$ . This compares with a mean rate of around  $8.6$  fF s $^{-1}$  during a 500 ms pulse.

### Capacitance changes elicited by a train of depolarizations

Figure 3 shows capacitance changes recorded in response to a train of ten depolarizations to +20 mV applied at a



**Figure 3. Effects of divalent cations on the capacitance response to a pulse train**

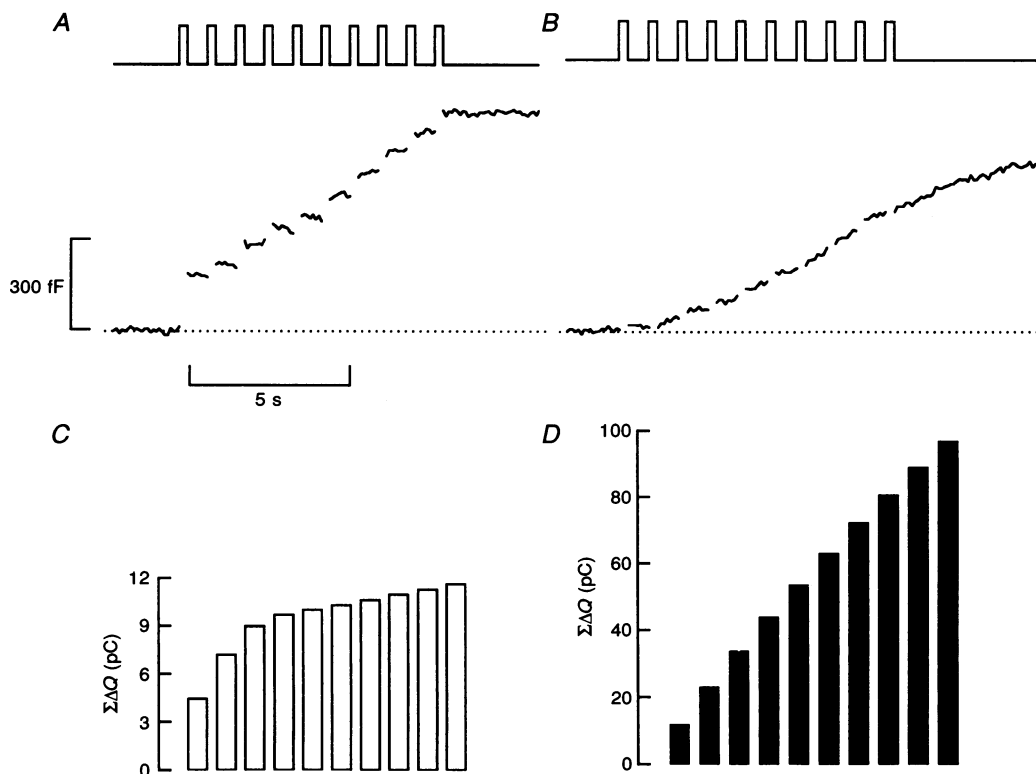
Representative recordings of exocytosis and endocytosis induced by a train of pulses in 5 mM each of  $\text{Ca}^{2+}$  (top),  $\text{Ba}^{2+}$  (middle) or  $\text{Sr}^{2+}$  (bottom). The train was composed of ten 200 ms pulses from  $-70$  to  $+20$  mV with an interpulse interval of 800 ms. The vertical dashed line indicates the cessation of the pulse train.

frequency of 0.5 Hz in either  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  or  $\text{Sr}^{2+}$  solution. It is noteworthy that, as in the case of a single pulse, exocytosis continues to increase after cessation of the train in  $\text{Ba}^{2+}$ , but not in  $\text{Sr}^{2+}$  or  $\text{Ca}^{2+}$  solution. This continuous increase was present in 80% of cells in  $\text{Ba}^{2+}$  solution and amounted to ~60% of the total response: the response during the train was  $241 \pm 22$  fF and the capacitance increase after the train was  $345 \pm 18$  fF ( $n = 18$ , all cells were included in this analysis). The total capacitance increase elicited by the depolarizing train was  $485 \pm 58$  ( $n = 8$ ),  $586 \pm 49$  ( $n = 18$ ) and  $69 \pm 15$  fF ( $n = 9$ ) for  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$ , respectively. The corresponding cumulative charge entry was  $33.4 \pm 6.1$  ( $n = 8$ ),  $186 \pm 35$  ( $n = 18$ ) and  $38.4 \pm 3.5$  pC ( $n = 9$ ), and exocytosis amounted to  $14.4 \pm 4.7$ ,  $3.2 \pm 1.1$  and  $1.8 \pm 0.9$  fF pC $^{-1}$ , in  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$ , respectively. Thus  $\text{Ca}^{2+} > \text{Ba}^{2+} > \text{Sr}^{2+}$  also when exocytosis is elicited by a train of depolarizations. A decrease in cell capacitance, reflecting membrane retrieval (endocytosis), was observed to follow exocytosis in the presence of all three divalent cations and is discussed more fully below.

A further effect of the divalent cation on the capacitance response to a depolarizing train is illustrated in Fig. 4. In ~70% cells tested in  $\text{Ca}^{2+}$  solution (17 out of 25), the largest response was produced by the first depolarization of the train and subsequent pulses evoked smaller capacitance

changes (Fig. 4A). It has been argued (Ämmälä *et al.* 1993b) that this phenomenon may result from the  $\text{Ca}^{2+}$  transient produced by the first pulse being sufficient to activate exocytosis maximally and largely deplete a readily releasable pool of secretory granules, and that subsequent pulses produce smaller responses because this granule pool is not fully refilled during the interpulse interval. An additional possibility, however, is suggested by the saturation of the cumulative charge entry during the train (Fig. 4C), which probably results from an increasing  $\text{Ca}^{2+}$ -dependent inactivation of the  $\text{Ca}^{2+}$  current (Plant, 1988). This observation suggests that the local  $\text{Ca}^{2+}$  concentration in the immediate vicinity of the release sites may not increase as much during the later pulses in the train, thereby contributing to the reduced release. Consistent with this idea, in all cases no depression of the capacitance response was observed in  $\text{Ba}^{2+}$  solutions (Fig. 4B), where the cumulative charge entry did not saturate (Fig. 4D). The latter phenomenon may be attributed to the fact that the inward current inactivates less when  $\text{Ba}^{2+}$  is the charge carrier (Fig. 1; Plant, 1988). Figure 4 also shows that the total influx of  $\text{Ba}^{2+}$  during a depolarizing train is much greater than that of  $\text{Ca}^{2+}$ .

Figure 5A compares the immediate capacitance increase evoked by the first and tenth pulse of a train in solutions containing  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$  and  $\text{Ba}^{2+}$ . For either pulse, exocytosis



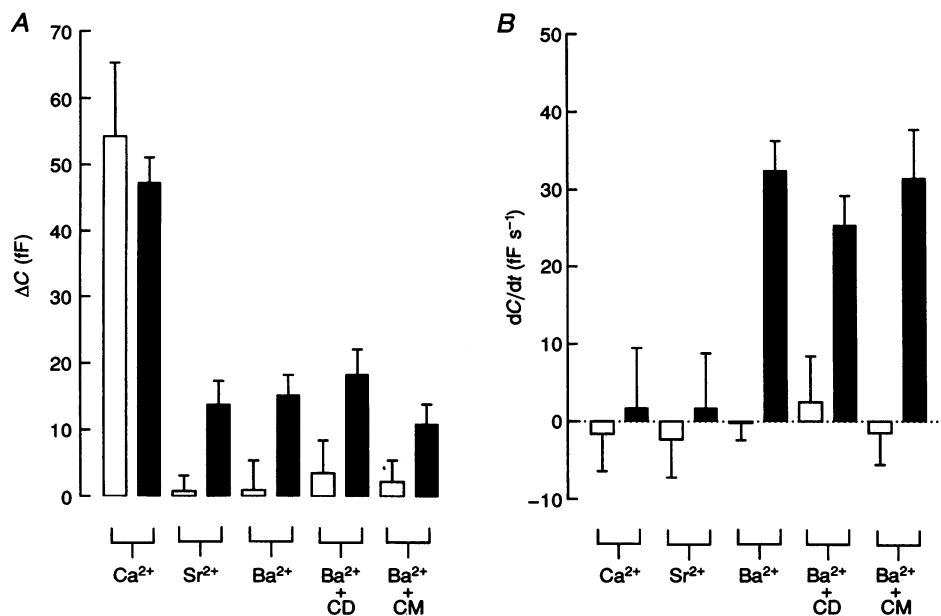
**Figure 4.** Capacitance changes and charge entry during a pulse train

Capacitance changes evoked by a train of ten 200 ms pulses from  $-70$  to  $+20$  mV in 5 mM  $\text{Ca}^{2+}$  (A) and 5 mM  $\text{Ba}^{2+}$  (B). Cumulative charge entry during the train, measured as the sum of the integrated inward currents in 5 mM  $\text{Ca}^{2+}$  (C) and 5 mM  $\text{Ba}^{2+}$  (D). Same cell as in A and B.

elicited in  $\text{Ba}^{2+}$  or  $\text{Sr}^{2+}$  solution was significantly smaller than that obtained in  $\text{Ca}^{2+}$  solution. However, whereas in  $\text{Ca}^{2+}$  solution the tenth pulse generally caused less exocytosis than the first, there was a very marked increase in the exocytotic response to the tenth pulse when  $\text{Ba}^{2+}$  or  $\text{Sr}^{2+}$  carried the inward current. Figure 4*B* also shows that in  $\text{Ba}^{2+}$  solution, the initial depolarization of a train invariably elicited a far smaller capacitance increase than subsequent pulses. The marked facilitation observed in  $\text{Ba}^{2+}$  solutions probably reflects the failure of  $[\text{Ba}^{2+}]_i$  to return to basal levels between depolarizations, resulting in summation of the  $\text{Ba}^{2+}$  transients and a progressive increase in the concentration of the cation at the exocytotic release sites. A similar argument has been used to account for facilitation of exocytosis in  $\text{Ca}^{2+}$  solutions in both pancreatic  $\beta$ -cells and other endocrine cells (Thomas, Surprenant & Almers, 1990; Augustine & Neher, 1992; Ammälä *et al.* 1993*b*). There are at least two explanations for the fact that in our experiments facilitation was far more common when  $\text{Ba}^{2+}$ , rather than  $\text{Ca}^{2+}$ , was the charge carrier. First, the cumulative charge entry during a train of pulses rapidly saturated in  $\text{Ca}^{2+}$  (Fig. 4*C*) but not in  $\text{Ba}^{2+}$  (Fig. 4*D*) solution, which may be expected to result in a greater accumulation of  $\text{Ba}^{2+}$  close to the cell membrane. Secondly,  $\text{Ba}^{2+}$  may not be buffered as rapidly or efficiently as  $\text{Ca}^{2+}$  by the  $\beta$ -cell.

In  $\text{Ca}^{2+}$  solution, exocytosis often did not continue after the first few depolarizations in the train and the capacitance trace remained flat during the interpulse interval: only following the sixth pulse was continuous exocytosis observed during the interpulse interval, and the rate was lower than that which occurred during the preceding depolarization (Fig. 4*A*). By contrast, in  $\text{Ba}^{2+}$  solution, most exocytosis took place after the voltage step, during the interpulse interval, and the rate of exocytosis continued to increase throughout the train (Fig. 4*B*). The rate of the capacitance increase during the 200 ms interval following the first pulse is compared with that during the first second after the final (tenth) pulse in Fig. 5*B*. There was little difference in the mean rate of exocytosis in either  $\text{Ca}^{2+}$  or  $\text{Sr}^{2+}$  solution. In  $\text{Ba}^{2+}$  solution, however, there was a substantial potentiation of the rate of secretion following the pulse train compared with that occurring during the first interpulse interval. These results may also be explained by assuming that the  $\text{Ca}^{2+}$  concentration close to the release site rapidly returns to its resting level on repolarization but that  $\text{Ba}^{2+}$  is removed far more slowly and continues to accumulate with successive depolarizations.

The maximum rate of exocytosis in  $\text{Ba}^{2+}$  solution was determined from the first derivative of the capacitance increase ( $dC/dt$ ) observed during, and following, a



**Figure 5**

*A*, change in cell capacitance ( $\Delta C$ ) evoked by the first and tenth pulse of a train measured in 5 mM  $\text{Ca}^{2+}$  ( $n = 7$ ), 5 mM  $\text{Ba}^{2+}$  ( $n = 18$ ), 5 mM  $\text{Sr}^{2+}$  ( $n = 9$ ), 5 mM  $\text{Ba}^{2+}$  + 10  $\mu\text{M}$  cytochalasin D (CD;  $n = 6$ ) and 5 mM  $\text{Ba}^{2+}$  + 20  $\mu\text{M}$  colcemid (CM;  $n = 6$ ). The train was composed of ten 200 ms pulses from  $-70$  to  $+20$  mV with an interpulse interval of 800 ms. *B*, the rate of the capacitance change ( $dC/dt$ ) observed during the interpulse interval following the first pulse of a train, and during the first second following the last pulse of the train, measured in 5 mM  $\text{Ca}^{2+}$  ( $n = 7$ ), 5 mM  $\text{Ba}^{2+}$  ( $n = 18$ ), 5 mM  $\text{Sr}^{2+}$  ( $n = 9$ ), 5 mM  $\text{Ba}^{2+}$  + 10  $\mu\text{M}$  cytochalasin D (CD;  $n = 6$ ), 5 mM  $\text{Ba}^{2+}$  + 20  $\mu\text{M}$  colcemid (CM;  $n = 6$ ). The train was composed of ten 200 ms pulses from  $-70$  to  $+20$  mV with an interpulse interval of 800 ms.

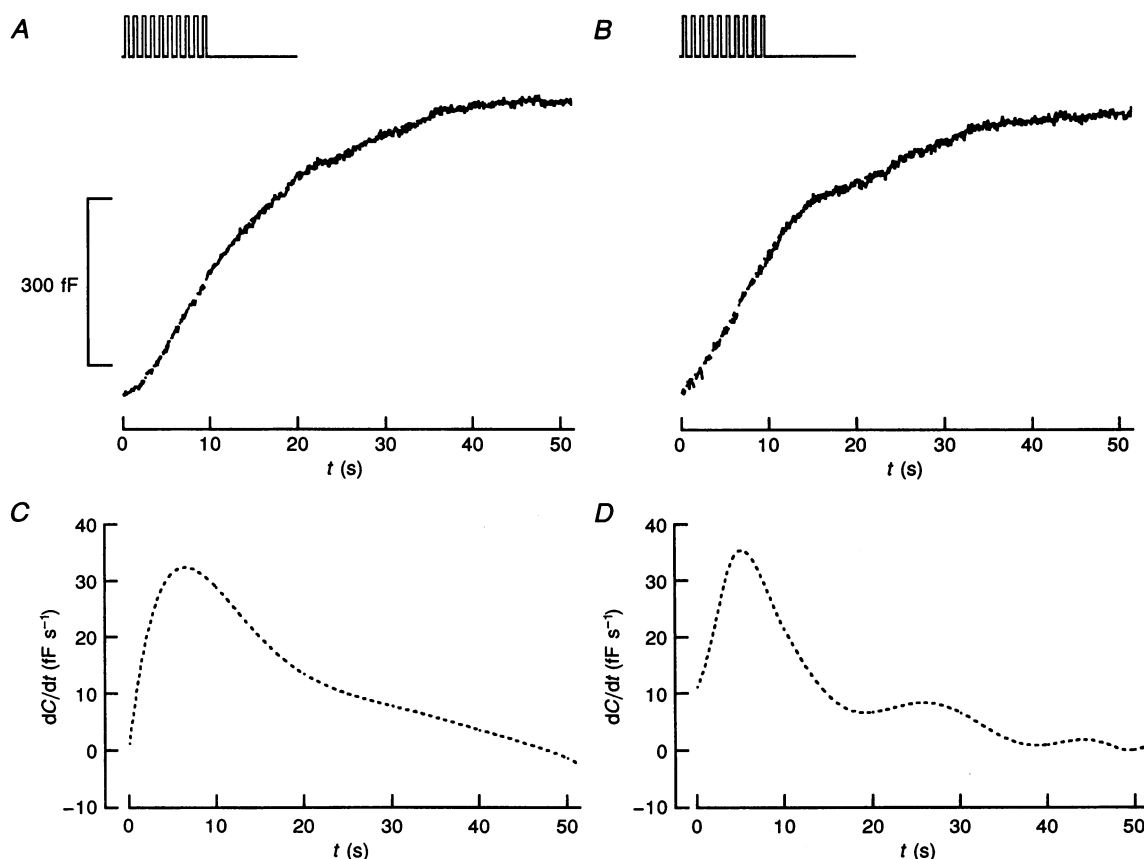
depolarizing train of pulses (Fig. 6). The data were smoothed before differentiation using a spline function. In about 20% of experiments, a single peak in  $dC/dt$  was observed (Fig. 6*A* and *C*). In most experiments, however, several peaks were present, occurring both within or following the train of pulses (Fig. 6*B* and *D*). The first peak was always largest and had a mean value of  $33 \pm 6 \text{ fF s}^{-1}$  ( $n = 18$ ), which corresponds to the maximum rate of exocytosis. This is about twice as fast as that found for the slow phase of the capacitance increase elicited by a single depolarization in  $\text{Ba}^{2+}$  solution ( $18 \text{ fF s}^{-1}$ ). The existence of oscillations in the rate of exocytosis may indicate the presence of several regulatory steps in the release process, or the existence of more than one population of vesicles. In about 15% of experiments, a negative endocytotic minimum within the train of pulses was found ( $dC/dt < 0$ ). Endocytosis is discussed more fully below.

#### Mechanisms underlying the slow phase of secretion in $\text{Ba}^{2+}$ solutions

We explored the possibility that the slow phase of  $\text{Ba}^{2+}$ -induced secretion involves the mobilization of secretory vesicles, using cytochalasin D to disrupt microfilaments

and colcemid to disrupt microtubules. It was difficult to obtain good seals after exposure to these agents. Therefore, in this series of experiments, the cell was first voltage-clamped using the perforated-patch configuration and then incubated without stimulation in a solution containing either of the agents which disrupt the cytoskeleton. Incubation times were 15 and 45 min for cytochalasin D ( $10 \mu\text{M}$ ) and colcemid ( $20 \mu\text{M}$ ), respectively. Following incubation, secretion was tested using a train of depolarizing pulses. The results are summarized in Fig. 5*A* and *B*. With cytochalasin D there was a slight increase in the amplitude and rate of the capacitance response to the first pulse of the train and a slight reduction in the rate of the capacitance change following a pulse train, but the data do not reach significance. There was no consistent effect of colcemid on either the magnitude or the rate of the capacitance changes. These results argue that microtubules and microfilaments are not involved in the slow phase of the exocytotic response in  $\text{Ba}^{2+}$  solution.

Another explanation for the second phase of exocytosis observed in  $\text{Ba}^{2+}$  solutions is that it results from  $\text{Ba}^{2+}$ -induced  $\text{Ca}^{2+}$  release from internal stores. To test this



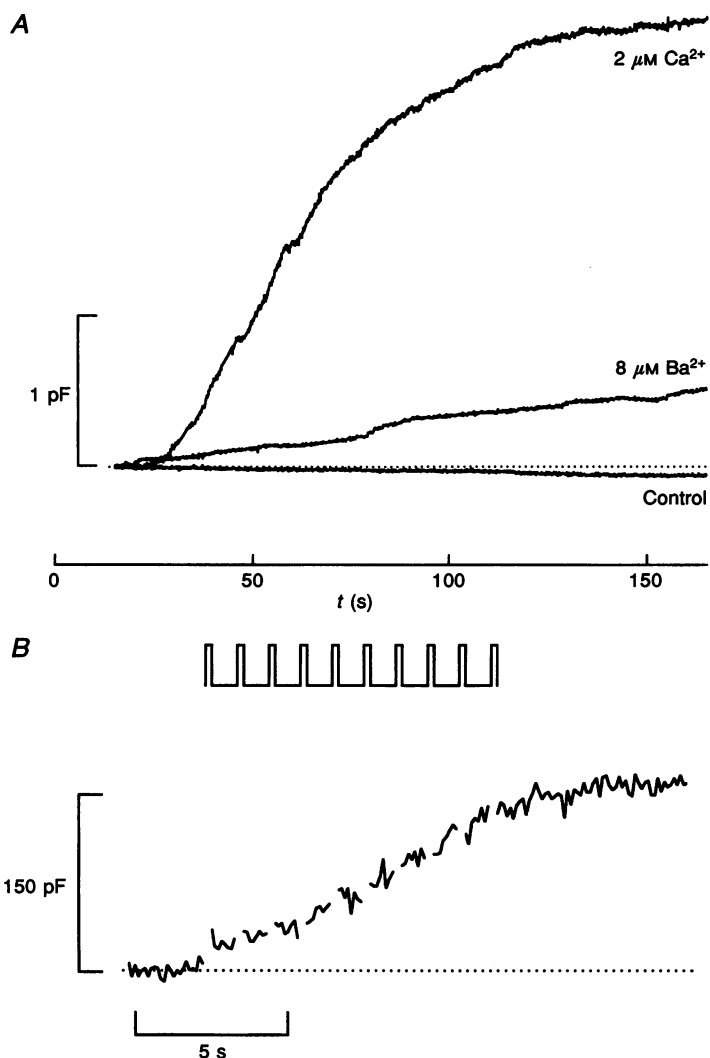
**Figure 6**

*A* and *B*, capacitance changes evoked by a train of ten 200 ms pulses from  $-70$  to  $+20$  mV in  $5 \text{ mM Ba}^{2+}$  in two different cells (*A* and *B*). The dotted lines through the capacitance record indicate the best polynomial fit to the data. *C* and *D*, Derivative of the polynomial fit to the data in *A* (*C*) and *B* (*D*).

possibility we compared the rate of exocytosis produced by infusion of the cell with  $8\ \mu\text{M}$  free  $\text{Ba}^{2+}$  or  $2\ \mu\text{M}$  free  $\text{Ca}^{2+}$ , using the standard whole-cell recording configuration. The intracellular divalent cation concentration was buffered with  $10\ \text{mM}$  EGTA to preclude any contribution from  $\text{Ca}^{2+}$  released from intracellular stores. As shown in Fig. 7A,  $2\ \mu\text{M}$   $\text{Ca}^{2+}$  caused a dramatic increase in cell capacitance after a short delay. A much smaller increase in exocytosis was observed for cells dialysed with  $8\ \mu\text{M}$   $\text{Ba}^{2+}$ . In cells dialysed with an intracellular solution containing no divalent cations there was either no change or a small decrease in exocytosis. The rate of the capacitance increase was measured over the steepest part of the curve and amounted to  $39 \pm 5\ \text{fF s}^{-1}$  ( $n = 10$ ) with  $2\ \mu\text{M}$   $\text{Ca}^{2+}$ ,

$1.1 \pm 0.2\ \text{fF s}^{-1}$  ( $n = 5$ ) with  $8\ \mu\text{M}$   $\text{Ba}^{2+}$  and  $-0.15 \pm 0.06\ \text{fF s}^{-1}$  ( $n = 6$ ) in control cells.

Further support for the idea that  $\text{Ba}^{2+}$  can directly stimulate exocytosis is provided by experiments like those shown in Fig. 7B, where we examined depolarization-mediated exocytosis in cells dialysed with  $10\ \text{mM}$  EGTA to buffer intracellular  $\text{Ca}^{2+}$  (calculated free  $\text{Ca}^{2+}$ ,  $< 2.3\ \text{nM}$ ) and with  $10\ \text{mM}$  extracellular  $\text{Ba}^{2+}$ . As is the case for  $\text{Ba}^{2+}$ -induced release in perforated-patch recordings (Fig. 4), the capacitance continued to increase between pulses. However, the total capacitance increase was smaller and the slow phase of exocytosis was much reduced. We attribute these differences to the high intracellular concentration of EGTA, which will buffer  $[\text{Ba}^{2+}]_i$ , although not as effectively as it



**Figure 7.** Capacitance changes evoked in whole-cell recordings

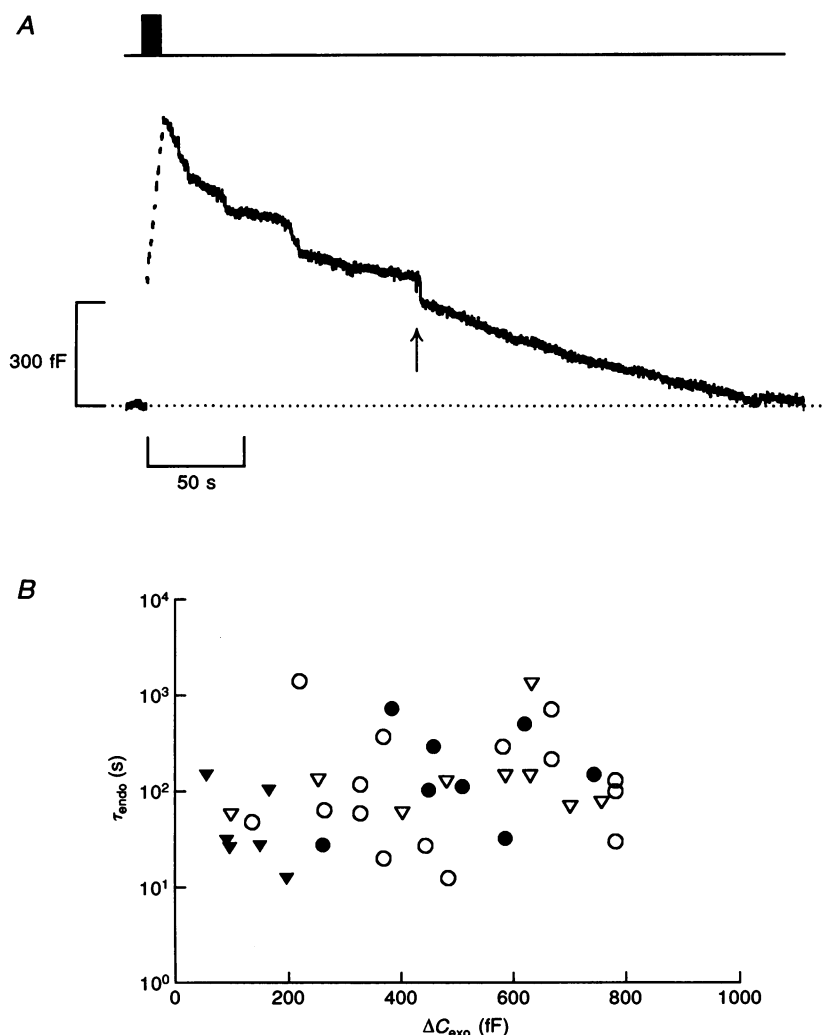
*A*, exocytosis initiated by infusion of the cell with control solution and with a solution containing  $8\ \mu\text{M}$  free  $\text{Ba}^{2+}$  or  $2\ \mu\text{M}$  free  $\text{Ca}^{2+}$ . Standard whole-cell recording. *B*, changes in cell capacitance evoked by a train of 200 ms depolarizations to  $+20\ \text{mV}$  from a holding potential of  $-70\ \text{mV}$  in the presence of  $10\ \text{mM}$  extracellular  $\text{Ba}^{2+}$ . Standard whole-cell recording with standard intracellular solution plus  $10\ \text{mM}$  intracellular EGTA.

does  $\text{Ca}^{2+}$  (2 mM intracellular EGTA is sufficient to completely inhibit depolarization-evoked exocytosis, Åmmälä *et al.* 1993b). These data indicate that  $\text{Ba}^{2+}$ -induced  $\text{Ca}^{2+}$  release from internal stores does not underlie secretion in  $\text{Ba}^{2+}$  solution.

We also investigated whether agents that elevate intracellular cyclic AMP are able to potentiate insulin secretion elicited by  $\text{Ba}^{2+}$ . As is the case for  $\text{Ca}^{2+}$  (Åmmälä *et al.* 1993), forskolin was able to potentiate insulin secretion evoked by  $\text{Ba}^{2+}$  ( $n=10$  cells) but the extent of this potentiation was very variable (data not shown). The slow phase of exocytosis was also present in the absence of agents that elevate cyclic AMP, indicating that it is independent of protein kinase A (PKA) activation.

### Endocytosis

In perforated-patch experiments a decrease in cell capacitance, attributable to endocytosis, was observed to follow exocytosis. In general, the rate and extent of membrane retrieval were far more variable than that of exocytosis. Thus, in some cells the capacitance declined continuously (Fig. 3), whereas in other cells large step decreases in endocytosis were observed. The step indicated by an arrow in Fig. 8A represents the retrieval of a membrane area of  $6.3 \mu\text{m}^2$ , equivalent to that of thirty-one secretory vesicles or a single vesicle of  $1.4 \mu\text{m}$  diameter. The presence of these large endocytotic steps was more common in cells with large inward currents, but was not obviously dependent on the divalent cation species. In



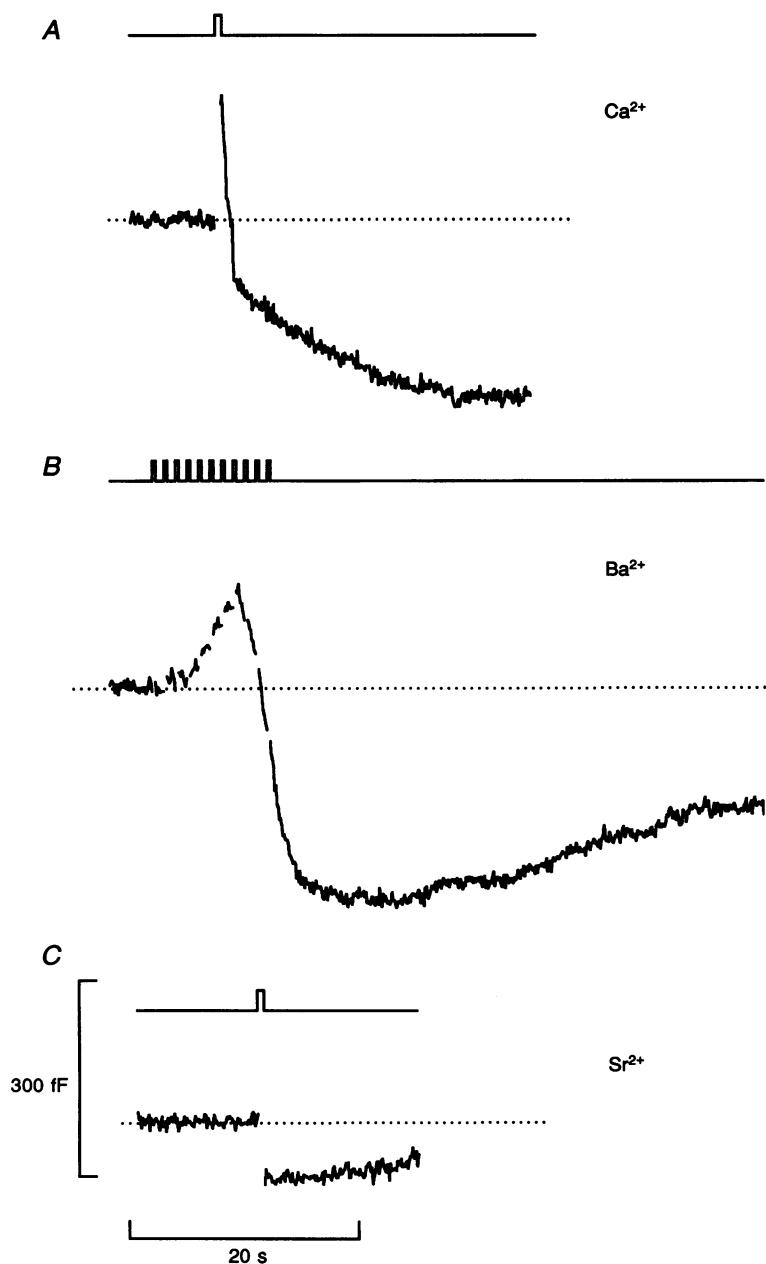
**Figure 8.** Effects of divalent cations on endocytosis

A, capacitance changes evoked by a train of ten 200 ms pulses from  $-70$  to  $+20$  mV in 5 mM  $\text{Ca}^{2+}$  solution. The period during which the train was applied is indicated above. The arrow indicates a capacitance decrease of 63 fF. B, time constants of endocytosis following a train of depolarizing pulses plotted against the total increase in membrane capacitance measured during the pulse train. The train consisted of ten 200 ms pulses from  $-70$  to  $+20$  mV applied at a frequency of 1 Hz. The extracellular solution contained 2.5 mM  $\text{Ca}^{2+}$  (○), 5 mM  $\text{Ca}^{2+}$  (●), 5 mM  $\text{Ba}^{2+}$  (▽) or 5 mM  $\text{Sr}^{2+}$  (▼).

5 mM  $\text{Ca}^{2+}$ , approximately 50% of cells showed distinct stepwise decreases in cell capacitance.

Endocytosis either began immediately following exocytosis or after a delay of several seconds. The divalent cation species did not noticeably alter the time at which endocytosis was initiated. In most cases, the amount of endocytosis was similar to that of exocytosis so that the cell capacitance returned to a value close to its original level (Figs 3 and 8). However, except for the abrupt decreases in cell capacitance discussed above, the rate of endocytosis was much slower than that of exocytosis. We have confined our analysis of endocytosis to those cells which showed a

smooth and continuous decrease in cell capacitance. We found that endocytosis in  $\text{Ca}^{2+}$  and  $\text{Sr}^{2+}$  solutions was better fitted by a single exponential than a straight line, but in 45% of experiments in  $\text{Ba}^{2+}$  solution the reverse was true. The initial rate (during the first 10 s) of endocytosis was  $3.5 \pm 0.7$  ( $n = 8$ ),  $3.4 \pm 0.7$  ( $n = 9$ ) and  $7.0 \pm 2.3 \text{ fF s}^{-1}$  ( $n = 6$ ), in  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solution, respectively. This compares with a mean exocytotic rate of 310, 98 and  $32 \text{ fF s}^{-1}$  in  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solution, respectively, measured in response to the same 500 ms pulse. The time constant for membrane retrieval varied from a few seconds to several minutes, and was not



**Figure 9. Endocytosis at elevated concentrations of divalent cations**

Examples of fast endocytosis measured in the presence of 20 mM each of  $\text{Ca}^{2+}$  (A),  $\text{Ba}^{2+}$  (B) or  $\text{Sr}^{2+}$  (C) in response to a single depolarization or a train of ten 200 ms pulses from  $-70$  to  $+20$  mV.

obviously related to the amount of preceding exocytosis (Fig. 8B). The mean time constant of endocytosis was  $243 \pm 88$  ( $n = 8$ ),  $234 \pm 137$  ( $n = 9$ ) and  $57 \pm 22$  s ( $n = 6$ ), in  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solution, respectively. The rate observed in  $\text{Sr}^{2+}$  solution was significantly different from that in  $\text{Ba}^{2+}$  solution ( $P = 0.95$ ).

### Effects of high divalent cation concentration

We further explored the relationship between exocytosis, endocytosis and divalent cations by examining secretion at an extracellular divalent cation concentration of 20 mM (Fig. 9). At high divalent cation concentration, the immediate increase in capacitance elicited by a single pulse was generally smaller than that observed with a divalent cation concentration of 5 mM, and the slow phase of secretion in  $\text{Ba}^{2+}$  solution was suppressed. The mean exocytosis elicited by a single pulse was  $1.4 \pm 1.4$  ( $n = 28$ ),  $0.05 \pm 0.01$  ( $n = 13$ ) and  $0.12 \pm 0.2$  fF  $\text{pC}^{-1}$  ( $n = 21$ ), in  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  solution, corresponding to 17, 5 and 17% of the response in 5 mM cation, respectively. In addition, a very fast decrease in cell capacitance was frequently observed to follow exocytosis, with time constants ranging from 100 ms (the sampling frequency) to a few seconds. This fast endocytosis was observed both in response to a single pulse or a train of pulses and with all three cation species (Fig. 9). The capacitance method measures membrane area, and so provides a sum of both exocytotic and endocytotic events. Thus, it is possible that the faster membrane retrieval observed at high divalent cation concentrations may partially, or fully, mask the exocytotic response and account for the smaller capacitance increase elicited by depolarization.

The amount of membrane retrieval was also potentiated at high divalent cation concentrations and endocytosis was sometimes observed without apparent exocytosis, or in excess of exocytosis (Fig. 9). A similar phenomenon has been observed in melanotrophs (Thomas, Lee, Wong & Almers, 1994). The apparent decrease in exocytosis observed at high divalent cation concentrations, coupled with the marked increase in the rate of endocytosis and the frequent occurrence of excess endocytosis, suggest that divalent cations may both potentiate and accelerate endocytosis. We have not attempted to analyse this phenomenon quantitatively because the increased rate of endocytosis suggests that measurements of cell capacitance may not distinguish between exocytosis and endocytosis in high divalent cation solutions.

## DISCUSSION

### Exocytosis

Our results show that the inward currents carried by  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  in response to depolarization are of similar size, but that the effects of these ions on exocytosis are very different. The immediate capacitance increase elicited by a single depolarization was largest in  $\text{Ca}^{2+} > \text{Ba}^{2+} > \text{Sr}^{2+}$ .

This suggests that both  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  are less effective at eliciting exocytosis than  $\text{Ca}^{2+}$ . In contrast to a previous report that the fusion of secretory granules from isolated islet cells could be induced only by  $\text{Ca}^{2+}$  and not by  $\text{Sr}^{2+}$  (Dahl & Gratzl, 1976), we found that both  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  were capable of supporting exocytosis, albeit at a far slower rate than  $\text{Ca}^{2+}$ .  $\text{Ba}^{2+}$  is also able to support exocytosis from chromaffin cells (Ruden, Garcia & Lopez, 1993).

A sustained increase in exocytosis was observed to follow the depolarizing step in  $\text{Ba}^{2+}$ , but not in  $\text{Ca}^{2+}$  or  $\text{Sr}^{2+}$  solutions. The origin of this second phase is unclear. It does not appear to result from  $\text{Ba}^{2+}$ -induced  $\text{Ca}^{2+}$  release from internal stores since it still occurred in the presence of intracellular  $\text{Ca}^{2+}$  buffers. There was also no evidence that recruitment of secretory vesicles underlies the slow phase of secretion, since inhibitors of both microfilaments and microtubules were without effect. The simplest explanation for the slow phase is that  $\text{Ba}^{2+}$  remains in the vicinity of the secretory granules longer than  $\text{Ca}^{2+}$ . This possibility is suggested by our finding that facilitation of the capacitance response during a train of depolarizations usually occurs in  $\text{Ba}^{2+}$  solution, but only rarely in  $\text{Ca}^{2+}$  solution. This facilitation is reminiscent of that observed with  $\text{Ca}^{2+}$  in other endocrine cells (Thomas *et al.* 1990; Augustine & Neher, 1992), where it has been attributed to the local accumulation of the ion at the release site. In our experiments accumulation appears to be enhanced when  $\text{Ba}^{2+}$  is the charge carrier. The larger  $\text{Ba}^{2+}$  influx we observe probably contributes to this effect. In addition, it is possible that  $\text{Ba}^{2+}$  is removed more slowly from the vicinity of the secretory granules, because of the greatly reduced affinity of the  $\text{Ca}^{2+}$  pump for  $\text{Ba}^{2+}$ , and consequently a much lower extrusion rate (Schilling, Rajan & Stobl-Jager, 1989; Wagner-Mann, Hu & Strueck, 1992). The inability of  $\text{Ba}^{2+}$  to activate calmodulin may account for at least part of the lower affinity of the  $\text{Ca}^{2+}$  pump for this ion (Klee, 1988). A slow phase of secretion is also occasionally observed in  $\text{Sr}^{2+}$  solution when the divalent cation concentration is increased to 20 mM (data not shown). This may indicate that the buffering capacity of the  $\beta$ -cell is also lower for  $\text{Sr}^{2+}$  than for  $\text{Ca}^{2+}$  (i.e.  $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}$ ).

It has been proposed that the decline in exocytosis observed during a train of depolarizations in  $\text{Ca}^{2+}$  solution results from the depletion of a readily releasable pool of vesicles since it is not associated with a decrease in the average cytosolic  $\text{Ca}^{2+}$  concentration (Augustine & Neher, 1992; Ammälä *et al.* 1993b). The ability of a further train of depolarizations to elicit exocytosis after an interval is ascribed to the replenishment of this pool of granules. Our results suggest that if this idea is indeed correct, neither microtubules nor microfilaments are involved in replenishment of the readily releasable pool since neither cytochalasin or colcemid prevented the ability of a second train of depolarizations to elicit exocytosis in  $\text{Ba}^{2+}$  solutions. It is possible that the readily releasable pool may not be a

physical entity but rather represent the number of granules that can be accessed by activator processes involved in exocytosis before other processes which tend to inhibit release are stimulated. An alternative hypothesis, however, is that depression is related to the  $\text{Ca}^{2+}$  concentration at the release sites and that this falls during a train of pulses due to inactivation of the  $\text{Ca}^{2+}$  channels. Since  $\text{Ca}^{2+}$  indicators only monitor the mean cytosolic  $\text{Ca}^{2+}$  they do not report faithfully the  $\text{Ca}^{2+}$  concentration beneath the membrane, close to the release sites, which may be considerably less. Indeed, the submembrane  $\text{Ca}^{2+}$  concentration is more likely to be related to the amount of  $\text{Ca}^{2+}$  entry during the voltage step. Our results favour this hypothesis since the amount of divalent charge entry falls off far more steeply during a train of depolarizations in  $\text{Ca}^{2+}$  solution, in which depression of exocytosis is observed, than in  $\text{Ba}^{2+}$  solution where depression is less evident and occurs far more slowly. Thus, decreased charge entry may contribute to the depression of release.

It is worth noting that the effects of external  $\text{Ba}^{2+}$  on exocytosis resemble those observed (Ämmälä *et al.* 1993b) with  $\text{Ca}^{2+}$  when  $\beta$ -cells are dialysed with the calmodulin-binding domain of CaM kinase II (residues 290–309) which is a potent calmodulin antagonist and a specific inhibitor of  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase (CaM kinase II). When CaM kinase II was inhibited, not only was the exocytotic response to a voltage step greatly reduced, but in 30% of cells there was also a slow increase in capacitance for several seconds after the depolarization. Since  $\text{Ba}^{2+}$  fails to activate calmodulin (Klee, 1988), and thus would not be predicted to activate CaM kinase II, it seems plausible that at least part of the lower efficacy of  $\text{Ba}^{2+}$  in sustaining exocytosis is related to its inability to activate CaM kinase II. Exocytosis was only reduced by 60% in the presence of the calmodulin-binding domain (Ämmälä *et al.* 1993b) implying that  $\text{Ca}^{2+}$  is also involved in other steps in the secretory process. The fact that the immediate exocytotic response to a voltage step is reduced by 88% in our experiments when  $\text{Ca}^{2+}$  was replaced by  $\text{Ba}^{2+}$  suggests that  $\text{Ba}^{2+}$  may not be able to substitute for  $\text{Ca}^{2+}$  as effectively in these other step(s). Activation of protein kinase A (PKA) potentiates exocytosis by acting at a late stage of the secretory process, subsequent to elevation of intracellular  $\text{Ca}^{2+}$  (Jones, Fyles & Howell, 1986; Ämmälä *et al.* 1993a). The ability of forskolin also to potentiate exocytosis induced by  $\text{Ba}^{2+}$  solutions suggests that PKA may influence a calmodulin-independent secretory step.

### Endocytosis

The amount of endocytosis was closely correlated with that of exocytosis at low divalent ion concentration, and in most cases the cell capacitance returned to a value close to its original level. Thus, it appears that the  $\beta$ -cell is able to recognize and retrieve the same amount of membrane that has been secreted, as is essential if the cell is to avoid

swelling. Since both exocytosis and endocytosis increase by a similar amount when  $\text{Ba}^{2+}$ , rather than  $\text{Ca}^{2+}$ , is the charge carrier, our results favour the idea that the extent of membrane uptake is not directly influenced by the divalent cation species. Rather, differences in the magnitude of endocytosis may be attributed to the effect of the divalent cation on exocytosis. The rate of endocytosis appeared to be about fourfold faster when  $\text{Sr}^{2+}$ , rather than  $\text{Ba}^{2+}$  or  $\text{Ca}^{2+}$ , was the charge carrier. This cation specificity differs from that of exocytosis and suggests that membrane retrieval may involve different processes from those of exocytosis. In support of this idea, our data indicate that CaM kinase II does not mediate the effects of  $\text{Ca}^{2+}$  on endocytosis, as it does for at least part of the secretory response (Ämmälä *et al.* 1993b). Since  $\text{Ba}^{2+}$  does not bind to calmodulin (Klee, 1988), it would not be expected to activate calmodulin-dependent enzymes such as CaM kinase II, or calmodulin-dependent protein phosphatases, such as calcineurin; yet  $\text{Ba}^{2+}$  supports endocytosis.

Membrane retrieval was accelerated, and excess endocytosis more commonly observed, when the divalent cation concentration was increased to 20 mM, supporting the idea that divalent cations are involved in membrane uptake. Triggered endocytosis has been reported to be independent of intracellular  $\text{Ca}^{2+}$  in chromaffin cells (Von Grafenstein & Knight, 1993) and of extracellular  $\text{Ca}^{2+}$  at the presynaptic bouton (Ryan, Reuter, Wendland, Schweizer, Tsien & Smith, 1993). However, large transient increases in intracellular  $\text{Ca}^{2+}$  produce rapid endocytosis both in melanotrophs (time constant ( $\tau$ ), 350 ms; Thomas *et al.* 1994) and in  $\beta$ -cells (Ämmälä *et al.* 1993b), similar to those found when  $[\text{Ca}^{2+}]_o$  is increased. Since  $\text{Ca}^{2+}$  is required for exocytosis, and endocytosis is dependent on exocytosis, the  $\text{Ca}^{2+}$  dependence of membrane retrieval might simply reflect that of secretion. However, our finding that the rate of endocytosis is influenced by the concentration of the divalent cation suggests that in  $\beta$ -cells membrane retrieval itself may require divalent cations. Unlike exocytosis, however, both  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  can substitute fully for  $\text{Ca}^{2+}$  in this process.

### Implications for insulin secretion

Finally, our results indicate that both  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  can support exocytosis of  $\beta$ -cell secretory granules. In unclamped cells, the marked enhancement of insulin secretion by  $\text{Ba}^{2+}$  (over  $\text{Ca}^{2+}$ ) cannot be attributed to a direct action on the secretory machinery, however, and is more likely to be due to a combination of a greater accumulation of  $\text{Ba}^{2+}$  in the vicinity of the secretory vesicles, coupled with an enhanced  $\text{Ba}^{2+}$  influx through voltage-gated  $\text{Ca}^{2+}$  channels (Smith *et al.* 1993) which results from the inhibitory effect of  $\text{Ba}^{2+}$  on the  $\text{K}_{\text{ATP}}$  channel (M. Takano & F. M. Ashcroft, unpublished observations).

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#### Acknowledgements

We thank the Wellcome Trust and the British Diabetic Association for support. We also thank Drs C. Ämmälä and P. Rorsman (Göteborg University, Sweden) for generously teaching us the capacitance technique.

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Received 5 December 1994; accepted 7 March 1995.